Studies on the Resissance to Tolerance Induction against Human IgG in DDD Mice. V. Insignificant Contribution of Suppressor Cells to the Age-Related Change in Tolerogen Susceptibility

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Studies on the Resissance to Tolerance Induction against Human IgG in DDD Mice. V. Insignificant Contribution of Suppressor Cells to the Age-Related Change in Tolerogen Susceptibility.

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INTRODUCTION

Susceptibility to tolerance induction differs among strains of mice[1~3]. In SJL/J and NZB mice which show immunological abnormalities and suffer from autoimmune diseases or spontaneous tumors, tolerance to heterologous serum proteins was shown to be hardly inducible with increasing age[3~7]. Few reports concern to the suppressor cells which may engage in the tolerance induction against protein antigens, although there are accumulating reports showing decline of suppressor T cell activity with age in cell-mediated immunity[8,9] or generation of immunoglobulin-producing cells[10,11]. One paper reported by Gershwin and Steinberg[12] showed that NZB/W F_1 mice became more susceptible to tolerance induction against bovine γ-globulin by repeated injections of concanavalin A which induced suppressor cells *in vitro*[13].

In the series of papers, we have reported that DDD mice show resistance to tolerance induction against human IgG(HGG) which become apparent with age of mice[14]. The resistance is mediated by some set of spleen T cells which have recently generated under the influence of radioresistant compornent(s) of the aged thymus[14] and mainly located in the spleen[15]. It has still remained to be explored whether the development of the tolerogen resistance could be attributed to the progressive loss of suppressor cells with age or not. Experimental results reported here suggest that the difference with age in tolerogen susceptibility is not mediated by suppressor cells. Their possible roles as regulators were discussed.

MATERIALS AND METHODS

Mice. Female weanling DDD mice were supplied from the Breeding Unit of the Institute of Medical Science, University of Tokyo. They were allowed free access to food and water.

Antigens. Human IgG(HGG) was obtained as Fr. II (Cohn ethanol fractionation) by the courtesy of American Red Cross and purified by means of DEAE-cellulose column chromatography, equilibrated and eluted with 0.01 M phosphate buffer at pH 7.8. HGG solution was adjusted to be isotomic by an addition of NaCl and stored at 4°C, in sterile conditions. The amount of HGG in each preparation was estimated photospectrometrically by the equation of

$E^{1\%}_{280nm}=15$. Horse red blood cells (HRBC) (Nippon Biotest Laboratories Incorporated, Tokyo) were used as a control antigen.

Tolerance induction. HGG solution (10 mg/ml) was centrifuged at 123,000 g for 180 min in a 65 fixed-angle rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Immediately after centrifugation, the top one third portion of the supernatant in each tube was used as tolerogenic material (tHGG). Tolerance was induced by intraperitoneal (i.p.) injection of tHGG at a dose of 0.05 mg per g of body weight. To induce tolerance in reconstituted mice, 1 mg of tHGG was injected i.p. into each recipient.

Challenge immunization. Immunological materials of HGG was prepared by heating HGG solution at 63°C for 120 min, followed by chilling in ice bath[2]. The aggregated material (aHGG) was homogenized by Teflon homogenizer immediately before injection. Mice were challenged intravenously (i.v.) with 0.5 mg of aHGG plus 10^8 *Bordetella pertussis* vaccine (kindly donated by Dr. Y. Sato, 1st Department of Bacteriology, National Institute of Health, Tokyo) and then i.p. with the same amount of aHGG 11 days later. A tenth ml of 20 percent of HRBC (v/v) were injected as a control antigen with aHGG solution.

Cell suspension and cell transfer. For preparing nucleated cells in cell transfer experiments, Eagle's minimum essential medium (MEM) buffered with 20 mM HEPES, adjusted to pH 7.2 by 0.15 N NaOH was used throughout the experiments. Thymus, spleen, mesentric lymph node and bone marrow cell suspensions were prepared following the method described previously[6]. Viable cells in each preparation ware counted by the dye exclusion test using 0.25% Trypan blue in saline and an appropriate number of viable cells was injected i.v. into each recipient irradiated at 850 rad immediately before cell transfer.

Removal of adherent cells from spleen cell population. Adherent cells in spleen cell suspension were removed by the Sephadex G-10 column method[16]. Briefly, spleen cells prepared in MEM containing 2% normal mouse serum (NMS-MEM) were passed through a Sephadex G-10 column which had been equilibrated with NMS-MEM. All the processes were carried out at room temperature. Normal mouse (DDD) serum was used instead of heterologous serum to avoid unknown effects on the induction to tolerance to HGG which might share antigenic determinants with heterologous immunoglobulins. The spleen cells deprived of A cells were designated as Sp(-A).

Enumeration of antibody-producing cells in spleen. Spleen cell suspension was prepared as mentioned above using balanced salt solution[17] instead of MEM. Cells secreting antibodies against HGG and HRBC were enumerated by the indirect hemolytic plaque assay as reported by Kaplan and Cinader[18].

Thymectomy. Thymectomy was performed on 5-to 6-week-old DDD mice. At the end of the experiment, all mice were autopsied to check the complete removal of thymic lobes. No thymic remnants were found in any mice used in the Results.

RESULTS

Age-related changes of tolerogen susceptibility in spleen helper cells. In the previous paper[14], it was reported that the tolerogen resistance to deaggregated HGG in DDD mice increased

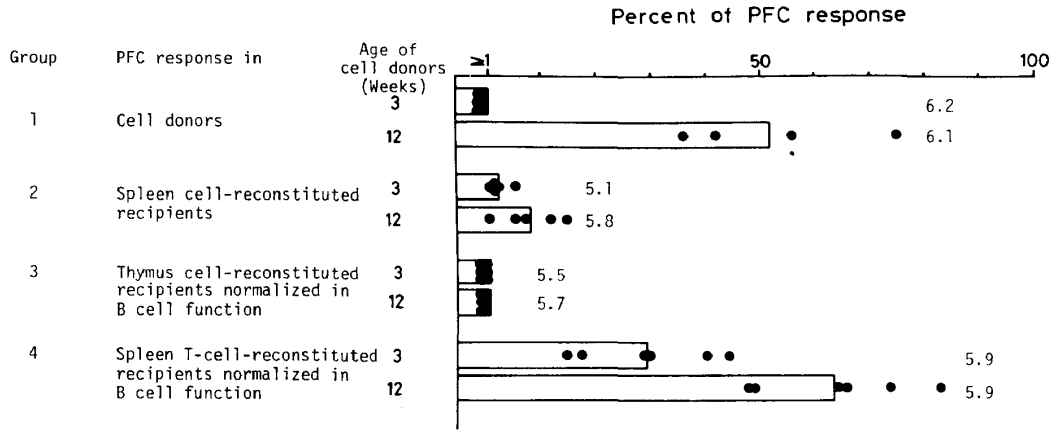

Fig. 1 Age difference of tolerance inducibility in whole animals, thymus, spleen and spleen T cells. A half of 3-week-old or 12-week-old DDD mice was tolerized and another half was left untreated as an immune control. Some of them were received challenge injections one week thereafter and tested for tolerogen suceptibility (group 1). Other mice became cell donors; spleen cells (5×10^7, group 2) or thymus cells (1×10^8) mixed with normal bone marrow cells (1×10^7, group 3) were injected i.v. into lethally irradiated syngeneic recipients. In group 4, spleen cells (5×10^7) were transferred with an excess number of normal bone marrow cells (1×10^7) in order to normalize B cell function in the recipients: this group refer to the tolerance inducibility in spleen T cells. Each circle refer to the percentage of response calculated by dividing the number of PFC in each recipient reconstituted with lymphoid cells from tolerized donors by the mean number of PFC in the recipients reconstituted with those from normal donors. Each bar shows a mean of the values, and was followed by log PFC per spleen of the immune control groups.

progressively between 3 and 7 weeks of age without the change in antibody responsiveness of control group against immunizations with aHGG and that the resistance in adult (12-week-old) animals were mediated by Tetron wool-non-adherent spleen-T cells. In the first experiment, the cellular sites of the resistance were examined. Tolerance inducibility in 3-week-old and 12-week-old DDD mice were compared as whole mice or in the cell transfer experiments in which lethally irradiated syngeneic mice were reconstituted with spleen cells, thymus cells or spleen cells plus normal bone marrow cells in order to normalize B cell function. Results are represented in Fig. 1. Tolerance was more profound (more than 99 percent suppression) in 3-week-old mice than that in 12-week-old mice (group 1 in Fig. 1). The age-dependent difference, however, was not observed in whole spleen cells when the cells from either tolerized or normal mice were transferred into the irradiated recipients (group 2 in Fig. 1). Previously it was shown that the resistance to tolerogen in 12-week-old mice was attributed to the nature of splenic T cells[14] rather than splenic B cells[19] or A cells[15]. The following experiments were focused on whether helper T cells in the central (thymus) or in the peripheral lymphoid organ (spleen) change with age in terms of the tolerogen susceptibility. Spleen cells from 12-week-old mice showed more resistance to tHGG than those from 3-week-old mice in the normalized B cell function of the transferred cells (group 4 in Fig. 1), while there was little difference in the tolerance inducibility between thymus cells from both donors (group 3 in Fig. 1). These results seem to indicate that the splenic T cells are responsible for the age-dependent changes in the tolerogen susceptibility.

Duration of tolerance. Next experiments were designed to see what happened if challenge

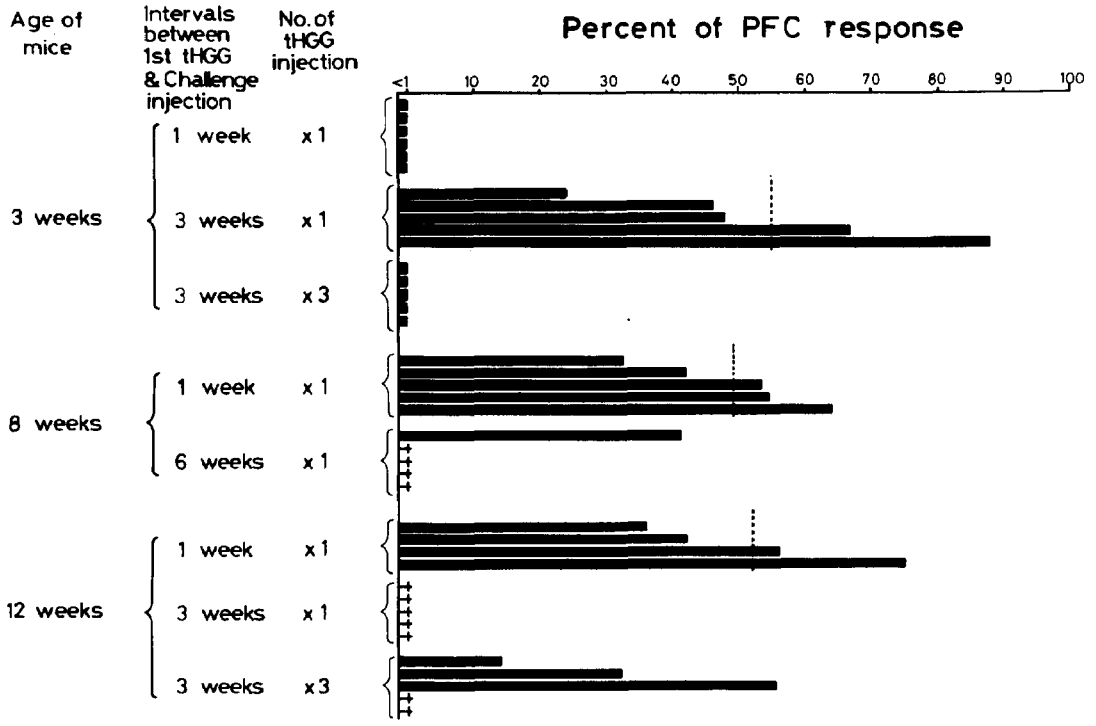

Fig. 2 Effect of varied intervals of tolerogen treatment on the hyporesponsiveness in 3- and 12-week-old DDD mice. Three- or 12-week-old mice treated with or without the tolerogen were further divided according to the indications shown in the second and the third columns in this figure. Percentage of response was calculated as mentioned in Figure 1. Each bar refers to each animal and a cross (+) indicates an animal died from anaphylaxis after the first challenge immunization. Dotted lines represent means of the percent of response.

immunization was made 3 weeks after the tolerogen injection instead of one week. As shown in Fig. 2, unexpected results were obtained in 12-week-old mice; all mice died immediately or within a few days after the challenge immunization. At postmortem examination, intestine and lung were congestive. This may suggest the production of enough amounts of antibodies to cause anaphylactic shock 3 weeks after the tolerogen injection. On the other hand, 3-week-old mice did not die and their reactivity to aHGG fairly recovered to a half of the controls.

The effect of repeated injections of tolerogen. Marked difference in tolerance inducibility was again shown between the ages of mice when tolerogen treatment was carired out by three weekly repeated injections (Fig. 2). The repeated injections induced again complete suppression in 3-week-old mice. On the other hand, in 12-week-old mice the same course of the treatments caused fatal anaphylactic shock in two out of five mice.

The effect of the removal of A cells on tolerance induction. We attempted to diminish the effect of A cells of donor origin on the tolerance induction and the challenge immunization by passing spleen cells through a Sephadex G-10 column immediately before cell transfer. Lethally irradiated mice were reconstituted with spleen cells (Sp(-A)) from either 3- or 12-week-old mice, and the third group of mice were transferred with the mixture of both types of cells. Tolerogen susceptibility was assessed and the results were presented in Table 1. Significant decrease in anti-HGG response by the tolerogen treatment was observed only in 3-week-old spleen cells (Sp_3(-A)).

Table. 1 Failure to demonstrate suppressive activity by spleen cells from 3-week-old DDD mice[a]

Cells transferred	tHGG	No. of recipients	$\log_{10}$ PFC±SD/spleen	R[b]	p
Sp_3 (−A)	−	5	5.16±0.12(145,000)[c]	100	0.008
	+	5	4.46±0.24(29,400)	20	
Sp_{12} (−A)	−	5	5.15±0.07(144,000)	100	>0.9
	+	5	5.25±0.10(180,000)	125	
Sp_3 (−A)+Sp_{12} (−A)	−	5	5.33±0.06(218,000)	100	>0.9
	+	5	5.01±0.19(103,000)	47	

a) Ten-week-old DDD mice, lethally irradiated, were reconstituted with spleen cells (5×10^7) from 3- or 12-week-old donors. These cells had been passed through a Sephadex G-10 column to remove A cells from the cell population. The third group of recipients was reconstituted with a mixture of spleen cells from the two different types of donors. Each group was further divided into two groups; one received tolerogen three days after the cell transfer and another was left untreated. One week after the tolerogen treatment, all mice were injected with the challenge antigens.

b) Percent of PFC response.

c) Figures in parentheses refer to geometric means of PFC per spleen.

Table. 2 Failure to demonstrate suppressive activity in spleen cells from profoundly tolerized DDD mice[a]

Cells transferred	$\log_{10}$ PFC±SD/spleen	R[b]	p
$Spleen_n$	5.41±0.21 (260,000)[c]	100	—
$Spleen_t$	3.42±0.14 (2,600)	1	0.008
$Spleen_n$+$Spleen_t$	5.77±0.47 (596,000)	229	0.31

a) Weekly repeated treatment with tolerogen started at 3 weeks of age. At the age of 6 weeks, the tolerized mice became cell donors. Lethally irradiated 10-week-old syngeneic mice were reconstituted i.v. with 5×10^7 spleen cells and they received the course of the challenge immunization one day thereafter. PFC per spleen was enumerated 4 days after the last injection of the antigen. Subscripts mean the origin of cells; t=tolerized mice and n=age matched normal mice.

b) Percent of PFC response.

c) Figures in parentheses refer to geometric means of PFC per spleen.

Table. 3 Unresponsiveness of tolerant spleen cells normalized in B cell function[a]

Cells transferred	anti-HGG		anti-HRBC	
	$\log_{10}$ PFC/spleen	R[b]	$\log_{10}$ PFC/spleen	R[b]
$Spleen_n$+Bone $marrow_n$	6.03±0.08 (1,070,000)[c]	100	5.99±0.11 (977,000)	100
$Spleen_t$+Bone $marrow_n$	4.29±0.28 (19,500)	2	5.86±0.10 (677,000)	69
	p=0.002		p=0.04	

a) Donor mice were pretreated with tolerogen under the same conditions as presented in Table 2. Eight-week-old, thymectomized mice were lethally irradiated and reconstituted with spleen cells (5×10^7) and an excess number of normal bone marrow cells (1×10^7). Two weeks later, the recipients were immunized i.p. with the mixture of aHGG (500 μg) and HRBC (4×10^8). This immunization was repeated two more times with a week interval. PFC per spleen was enumerated 4 days after the last injection of antigens. Subscripts mean the origin of cells; t=tolerized mice and n=age matched normal mice.

b) Percent of PFC response.

c) Figures in parentheses refer to geometric means of PFC per spleen.

Failure to demonstrate suppressive effects by spleen cells from normal or tolerized young mice. Next experiment was carried out to examine whether suppressor cells were generated or not in tolerance induced by the repeated injections of tolerogen as shown in the previous experiments. Three-week-old mice were treated with three weekly injections of tolerogen and they became cell donors at the age of 6 weeks. Spleen cells from these mice were unresponsive to the challenge immunization in the adoptively transferred hosts. This hyporesponsiveness was not mediated by suppressor cells because no reduction in the number of antibody-producing cells was observed in the recipients transferred with the mixture of tolerized and normal spleen cells (Table 2). In addition, the hyporesponsiveness of the tolerized spleen cells normalized in B cell function by the addition of an excess number of bone marrow cells lasted as long as 5 weeks (Table 3). These data suggest that the tolerant state might be due to clonal unresponsiveness and not due to an active suppression.

Immunosuppressive activity of thymus cells. As mentioned above, thymus cells are very sensitive to the tolerogen treatment (Fig. 1). Then, it was investigated whether thymus cells from tolerized mice contain suppressor cells. Thymus cells from either normal or tolerized 4-week-old mice, or the mixture of them were transferred with normal bone marrow cells into irradiated recipients, then challenged and ant-HGG responses were tested. The results were indicated in Table 4. Data show a significant reduction (85 percent) in the number of antibody-producing

Table. 4 Immunosuppressive activity of thymus cells from tolerized mice[a)]

Cells transferred	No. of recipients	$\log_{10}$ PFC±SD/spleen	R[b)]	p
Thy_n+BM_n	4	5.54±0.35 (349,000)[c)]	100	—
$Thy_n+Thy_t+BM_n$	6	4.74±0.17 (52,000)	15	0.016
Thy_t+BM_n	4	2.59±0.42 (394)	0.1	0.028

a) Lethally irradiated mice were reconstituted with bone marrow cells (1×10^7) and thymus cells (5×10^7) from 4-week-old normal or mice tolerized one week before they became cell donors. The number of PFC per spleen was enumerated 4 days after the second immunization of the routine schedule for challenge injections. Subscripts mean the origin of cells; t=tolerized mice and n=age matched normal mice.
b) Percent of PFC response.
c) Figures in parentheses refer to geometric means of PFC per spleen.

Table. 5 Immunosuppressive activity of normal thymus cells[a)]

Cells transferred	No. of recipients	$\log_{10}$ PFC±SD/spleen	R[b)]	p
LN+BM	6	4.31±0.09 (20,600)[c)]	100	—
LN+Thy+BM	6	3.58±0.47 (3,800)	18	0.008
Thy+BM	6	4.22±0.25 (16,000)	80	0.994

a) Ten-week-old mice, lethally irradiated, were reconstituted with bone marrow cells (1.5×10^7) and lymph node cells (1×10^7) and/or thymus cells (1×10^8) from 10-week-old, normal mice. The first immunization was carried out on the day of the cell transfer. A half of each group was treated with or without re-challenge immunization on the day 4. Since no difference in the number of PFC per spleen between the two subgroups was obtained on the day 8 of the first immunization, figures in this table represent mean numbers of PFC in reference to the groups of cells transferred.
b) Percent of PFC response.
c) Figures in parentheses refer to geonetric means of PFC per spleen.

Table. 6 No effect of thymus cells on the tolerance induction in 12-week-old lymph node cells[a]

Exp.	Cells used for reconstitution	tHGG treatment	$\log_{10}$ PFC±SD/spleen	Percent of response and comparison between groups (p)		
1	Thy_4+BM_4	−	5.61±0.16(407,000)[b]	100		
		+	4.18±0.23(15,100)	3.7	*0.039*	
	$Thy_{12}+BM_{12}$	−	5.15±0.30(141,000)	100		
		+	2.37±0.66(243)	0.1	*0.003*	
2	$LN_{12}+BM_{12}$	−	5.39±0.21(245,000)	100		
		+	5.25±0.27(178,000)	63	*0.99*	
	$Thy_{12}+BM_{12}$	−	5.30±0.15(200,000)	100		100
		+	4.20±0.35(15,900)	7.9	*0.008*	8.9 *0.008* *0.31*
	$LN_{12}+Thy_{12}+BM_{12}$	+	5.00±0.30(100,000)			56 *0.016*

a) Lethally irradiated DDD mice were reconstituted with intravenous inoculation of bone marrow cell (1 or 1.5×10^7) plus thymus cells (1×10^8) and/or lymph node cells (1×10^7). Tolerance was induced immediately after the cell transfer. Subscripts mean age of donors in weeks.

b) Figures in parentheses reper to geometric means of PFC per spleen.

cells per spleen in mice reconstituted with normal thymus cells plus bone marrow cells by an addition of thymus cells from tolerized mice. To test whether the suppressive activity of thymus cells was introduced only by the tolerogen pretreatment, thymus cells from adult normal mice (10-week-old) were mixed with bone marrow cells and lymph node cells from the same aged donors and injected into lethally irradiated mice. Antibody-producing cells were reduced in mice reconstituted with lymph node cells and bone marrow cells by the addition of thymus cells. Thus, almost the same degree of suppressive activity was observed in normal thymus cells from 10-week-old donors (Table 5) as in tolerized thymus cells.

No effect of thymus cells on the tolerance induction in 12-week-old lymph node cells. The last experiment was designed to see the effect of thymus cells on the tolerance induction in lymph node cells from 12-week-old donors. Complete tolerance was shown to be introduced in both thymus cells from 3- and 12-week-old mice (Table 6, experiment 1). Helper T cells in lymph node are obstinately resistant to the tolerance induction in this experimental schedule as compared with those in thymus, despite both of them contained the same degree of helper activity against immunogenic forms of HGG (Table 6, experiment 2). An attempt was made to see whether the tolerogen sensitive thymus cells make lymph node cells sucecptible to the tolerogen treatment. Mixtures of 1×10^7 lymph node cells, 1×10^8 thymus cells and 1.5×10^7 bone marrow cells from 12-week-old donors were inoculated into 10-week-old, lethally irradiated mice. Then, tolerance inducibility was compared with that in mice reconstituted with either lymph node cells or thymus cells from age-matched donors (Table 6, experiment 2). The group of mice reconstituted with both thymus cells and lymph node cells could not be rendered hyporesponsive against the challenge immunization. These results may suggest that tolerogen susceptible thymus cells do not facilitate the induction of tolerance and that the immunosuppressive potency of thymus cells probably decayed before the challenge injection of aHGG into the reconstituted animals.

DISCUSSION

In the previous paper of this series of studies[14], it has been shown that the degree of tolerance

to HGG in DDD mice is an age-dependent phenomenon and that the resistance develops rapidly in the first two months after birth. The accellerated development in the tolerogen resistance could also be observed in SJL/J[5~7] and NZB[3,4,7] mice which show immunological abnormalities. We have investigated the mechanisms of the tolerogen resistance which may be helpful for the understanding of the development of autoimmunity. NZB mice have widely used as one of the animal models of human autoimmune diseases[20,21]. One of the outstanding immunological characteristics of the mice is a high rate of decline of immunosuppressive or immunoregulatory activities of T cells[8,10,22]. Although relationships between tolerance induction to deaggregated serum proteins and production of suppressor cells have been suggested by many workers based on their experimental results using normal strains of mice[23~31], presence or absence of significant contribution of suppressor cells in the tolerogen resistant strains has remained to be explored.

Recent report of Cinader *et al.*[29], using SJL/J mice, showed that the level of immunosuppressive activities in intact thymus cells was lost completely at around 13 weeks of age. It is of interest to see whether in SJL/J the suppressor cells is required in the induction and the termination phase of tolerance, because the treatment of SJL/J mice with deaggregated xenogeneic IgG caused priming in aged mice[32] and their spleen cells[6].

In the present report, we attempted further to investigate whether the tolerance induction in DDD mice is mediated by suppressor cells and if so, whether the age-related decline of the suppressive activity is solely attributed to the tolerogen resistance. As shown in Table 2, no suppressive activity was demonstrated in spleen cells from profoundly tolerized mice received weekly repeated injections of tolerogen and almost the same degree of tolerant state was observed in both intact mice and mice reconstituted with spleen cells from tolerized donors. In the further experiments, mixing young spleen cells with tolerogen-resistant 12-week-old spleen cells could not help the latter to be rendered tolerant (Table 1). At this end of experiments it could be concluded that tolerance induced in 3-week-old DDD mice, at least in spleen cells, is not mediated by suppressor cells.

In contrast, thymus cells in which profound suppression was introduced irrespective of *in situ* or in irradiated mice, did express suppressive activities when they were stimulated with the immunogen. Thus, thymus cells from tolerized, 4-week-old mice showed significant suppressive activity (Table 4). However, this activity could not be introduced by the treatment with tolerogenic HGG, because almost the same degree of suppression was observed in thymus cells even from more aged, or 10-week-old mice (Table 5). We did not yet have direct observations about the type of suppression; whether they are antigen specific or non-specific. In the antibody response to hapten-modified syngeneic spleen cells, Lawrence and Weigle[33] showed that thymus cells from normal mice contained suppressor cells and that their activity could be expressed at a relatively high dose in the cell recepients. Presence of suppressor cells in tolerized thymus cells but not in bone marrow cells does not consist with the results of Weigle *et al.*[26] who could find suppressive activities in the latter. This controversy may be from the different experimental system used by them; tolerance induction against carrier fowl IgG and the carrier-primed immune system to which the suppressive activity was assessed. Types of preparations of HGG could also give rise to different experimental results in terms of induction of suppressor cells[31], and it has been reported that

much more effective immunosuppresisve spleen cells were produced by tHGG solution ultracentrifuged in an angle-fixed rotor than that in a swing-out rotor[24]. Tolerogen preparation in the former applied in our experiment here, seems to contain less contamination of aggregated forms of HGG[24].

To test a possible role of suppressor cells in the tolerance induction, it has remained to be examined if cells which could be rendered tolerant and be able to suppress antibody responses make peripheral lymphoid cells easier to be tolerized. An attempt to induce tolerance in 12-week-old lymph node cells transferred with thymus cells resulted in failure to promote hyporesponsiveness (Table 6), suggesting that the immunosuppressive effect of thymus cells did not engage in the early events of tolerance induction in the peripheral lymphoid organs and also that the suppressive effect might disappear within a week after cell transfer. It may be concluded that the age-related changes of tolerance inducibility in DDD mice to extremely deaggregated HGG by ultracentrifugation in a fixed angle rotor is not mediated by suppressor cells. According to accumlating reports[23~31], the degree of unresponsive or hyporesponsive state is not always associated with the generation of suppressor cells. Antigen specific suppressor cells could also be generated in immunized animals[34] or in spleen cells immunized in *in vivo* culture system[35]. It is most likely that an induction of tolerance and generation of suppressor cells is probably independent, though accompanying, immunological phenomena and that the age-dependent changes of the tolerance inducibility attributed to those of cells other than suppressor cells.

SUMMARY

Using DDD mice which were shown to aquire tolerogen resistance from 3 to 7 weeks of age, it was investigated whether suppressor cells contributed to the change with age in the tolerance inducibility to human IgG (HGG). Thymus cells from 12-week-old mice were as susceptible as those from 3-week-old mice to tolerance induction against HGG whereas splenic helper T cells of the former were resistant to tolerance induction. This suggests that the age-related change of the tolerogen susceptibility of whole mice may be reflected by that of splenic T cells. Suppressive activity was evaluated in cell transfer experiments and it was shown that spleen cells from either 3-week-old normal or profoundly tolerized mice lacked suppressor activity. Thumus cells from 3-week-old tolerized mice bore suppressive activity which was also shown in normal thymus cells, even in those from aged donors. It was also shown that tolerance inducibility to lymph node cells was not affected by the addition of thymus cells in cell transfer experiments. From the experimental results it was suggested that suppressor cells played little role in tolerance induction to HGG and the age-related increase in the resistance to tolerance inuction was not due to loss of suppressors.

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